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Loss of p19^{ARF} Eliminates the Requirement for the pRB-Binding Motif in Simian Virus 40 Large T Antigen-Mediated Transformation

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At least three domains of simian virus 40 large T antigen (TAg) participate in cellular transformation. The LXCXE motif of TAg binds to all members of the retinoblastoma protein (pRB) family of tumor suppressors. The N-terminal 70 residues of TAg have significant homology to the J domain of Hsp40/DnaJ and cooperate with the LXCXE motif to inactivate the pRB family. A bipartite C-terminal domain of TAg binds to p53 and thereby disrupts the ability of p53 to act as a sequence-specific transcription factor. The contribution of these three domains of TAg to cellular transformation was evaluated in cells that contained inactivating mutations in the pRB and p53 pathways. Cells that stably expressed wild-type or selected mutant forms of TAg were generated in mouse embryo fibroblasts (MEFs) containing homozygous deletions in the *RB*, *INK4a*, and *ARF* loci. It was determined that the J domain, the LXCXE motif, and the p53-binding domain of TAg were required for full transformation of wild-type and *RB*$^{-/-}$ MEFs. In contrast, *INK4a*$^{-/-}$ MEFs that lacked expression of p16^{INK4a} and p19^{ARF} and *ARF*$^{-/-}$ MEFs that lacked p19^{ARF} but expressed p16^{INK4a} acquired anchorage-independent growth when expressing wild-type TAg or mutant derivatives that disrupted either the pRB-binding or p53-binding domain. The expression and function of the pRB family members were not overly disrupted in *ARF*$^{-/-}$ MEFs expressing LXCXE mutants of TAg. These results suggest that inactivating mutations of p19^{ARF} can relieve the requirement for the LXCXE motif in TAg-mediated transformation and that TAg may have additional functions in transformation.

Simian virus 40 (SV40) large T antigen (TAg) has been used extensively as a model system to study cellular transformation. TAg has the ability to transform a wide variety of normal cells seemingly by affecting the functions of a small number of cellular proteins. To transform wild-type (WT) mouse embryo fibroblasts (MEFs), TAg utilizes at least three domains: the J domain, the LXCXE motif that binds to the retinoblastoma protein (pRB) family of proteins (pRB, p107, and p130), and the p53-binding domain (12, 13, 17, 20, 65, 77, 81). The J domain is a highly conserved element present in all members of the DnaJ/Hsp40 family of molecular chaperones as well as all polyomavirus T antigens (39). DnaJ proteins bind specifically to hsp70 homologues to perform various chaperone activities, including the destruction of specific proteins (reviewed in reference 63). The J domain of TAg binds to hsc70 and participates in the inactivation of pRB family members (27, 56, 60, 68, 69). The J domain and the LXCXE motif of TAg cooperate to disrupt the ability of pRB family members to repress E2F-dependent transcription and to decrease the levels of hyperphosphorylated p107 and p130 (27, 60, 64, 65, 68, 69, 77). Thus, the J domain and LXCXE motif of TAg appear to induce transformation and promote cell growth by interfering with the functions of pRB, p107, and p130. The p53-binding domain of TAg binds to the specific DNA-binding domain of p53, thereby directly interfering with the ability of p53 to activate transcription (3, 21, 51). Therefore, it is believed that TAg can transform cells primarily by interfering with p53 and the pRB family.

The original observations that TAg binds to p53 and the pRB family set into motion a large field of research that has led to a more complete understanding of the role of these tumor suppressors in the development of cancer (13, 44, 46). Not only is pRB itself mutated in a wide variety of cancers, wild-type pRB can be functionally inactivated by expression of an LXCXE-containing viral oncoprotein. pRB can also be inactivated by hyperphosphorylation as a result of overexpression of cyclin D1 or loss of expression p16^{INK4a}. p16^{INK4a} binds to cdk4 and blocks the association of cdk4 with D-type cyclins (57). Overexpression of p16^{INK4a} in *RB*$^{+/+}$ cells prevents phosphorylation and subsequent inactivation of pRB by cyclin D1-cdk4 and promotes a cell cycle arrest in G_1 (41, 48). In contrast, p16^{INK4a} is unable to induce a cell cycle arrest in *RB*$^{-/-}$ cells, suggesting that the pRB pathway is required for p16^{INK4a}-mediated cell cycle arrest (48). p16^{INK4a} may also affect the phosphorylation status of p107 and p130, as each of these proteins migrates as a lower-phosphorylation form in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis after p16^{INK4a} overexpression (74; J. Zalvide and J. A. DeCaprio, unpublished observations). Therefore, p16^{INK4a} may regulate all three members of the pRB family.

In addition to p16^{INK4a}, the *INK4a* locus encodes a second gene product, p19^{ARF} (54). While each of these proteins is encoded by a unique first exon, the second and third exons encode p19^{ARF} in an alternate reading frame than p16^{INK4a} (54). It has been demonstrated that p19^{ARF} binds and inactivates MDM2 (36, 52, 67, 80). Since MDM2 contributes to the degradation of p53, overexpression of p19^{ARF} can result in an increased amount of p53, leading to an arrest in G_1 (28, 80). Therefore, through the expression of p16^{INK4a} and p19^{ARF}, the *INK4a* locus apparently participates in the regulation of both the pRB and p53 pathways.

Although TAg-mediated transformation is thought to involve primarily the inactivation of pRB family members and

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p53, studies of mice that contain mutations in the *INK4a* locus would seem to indicate that the functional inactivation of pRB and p53 is not sufficient to cause cellular transformation. *INK4a* knockout mice lack the second and third exons of the *INK4a* locus and do not express $p16^{INK4a}$ or $p19^{ARF}$, although they may express a truncated version of $p19^{ARF}$ that may be partially active (5, 53, 58). Despite this interference with both the p53 and pRB pathways, $INK4a^{-/-}$ MEFs were not transformed. Although they readily became immortalized, $INK4a^{-/-}$ MEFs were unable to grow in soft agar and required the expression of activated H-*ras* to become transformed (37, 58). $INK4a^{-/-}$ mice do have an increased incidence of several types of tumors including lymphomas and fibrosarcomas, which presumably occur after the mice receive additional genetic mutations (58). Curiously, the $ARF^{-/-}$ mice that fail to express $p19^{ARF}$ but retain expression of WT $p16^{INK4a}$ display a similar range of tumors as the $INK4a^{-/-}$ mice that have lost both $p19^{ARF}$ and $p16^{INK4a}$ (35, 37). In addition, the $ARF^{-/-}$ MEFs were readily immortalized and could be transformed by activated H-*ras* alone similarly to the $INK4a^{-/-}$ MEFs (37). Therefore, at least in the mouse model, it has been difficult to observe the specific contribution of $p16^{INK4a}$ to tumor suppression since the $INK4a^{-/-}$ and $ARF^{-/-}$ knockouts result in a similar phenotype.

Given that disruption of the *INK4a* locus was not sufficient to induce transformation of MEFs, the pRB and p53 pathways may retain some of their tumor suppressor activity in $INK4a^{-/-}$ cells. Indeed, it has been noted that p53 can be activated in the $ARF^{-/-}$ MEFs by ionizing radiation but not by adenovirus E1A (14, 37). It is thus possible that p53 may retain the ability to act as a tumor suppressor under some conditions in the $ARF^{-/-}$ and $INK4a^{-/-}$ mice. Alternatively, there may be tumor suppressor pathways other than pRB and p53 that retain activity in the $INK4a^{-/-}$ or $ARF^{-/-}$ MEFs. We wished to determine whether the loss or functional inactivation of tumor suppressor genes that are targeted by TAg would abrogate the need for the J domain, the LXCXE motif, or the p53-binding domain in TAg-mediated transformation. Using this approach, it had been previously demonstrated that the LXCXE motif was required to transform $RB^{-/-}$ MEFs, suggesting that p107 and p130 were also targeted by the LXCXE motif (11, 76). To identify additional pathways that may be targeted by SV40 TAg, we generated WT, $RB^{-/-}$, $ARF^{-/-}$, and $INK4a^{-/-}$ MEFs that stably expressed WT or selected mutants of TAg. We observed that TAg could fully transform MEFs from each of these genetic backgrounds. Notably, the LXCXE motif was absolutely required for transformation of WT and $RB^{-/-}$ MEFs but was dispensable for anchorage-independent growth of $ARF^{-/-}$ and $INK4a^{-/-}$ MEFs, suggesting that the pRB pathway was functionally inactivated by loss of $p19^{ARF}$. However, pRB family members were expressed normally, and their ability to repress E2F activity was intact in the $ARF^{-/-}$ and $INK4a^{-/-}$ MEFs expressing the LXCXE mutants of TAg. These results suggest the possibility that TAg's LXCXE motif may functionally inactivate other growth suppressors in addition to the pRB family and that $p19^{ARF}$ may have activity beyond the regulation of MDM2 and p53.

MATERIALS AND METHODS

Plasmids. The SV40 large TAg cDNA expression plasmids pSG5-T, pSG5-H42Q, pSG5-K1, pSG5-C105G, and pSG5-PVU-1 have been previously described (69, 76). pSG5-*dl*434-444 was cloned by substituting the *Pfl*MI-*Pst*I fragment from *dl*434-444 containing the mutant p53-binding site into pSG5-T (40). pSG5-HQ-K1 was similarly cloned by substituting the *Pfl*MI-*Pst*I fragment of pSG5-K1 into pSG5-HQ. pSG5-T1-135 and pSG5-T1-350 were generated by PCR amplification of a TAg cDNA with the appropriate primers. DNA sequencing confirmed the identity of all constructs.

The p21 promoter-luciferase reporter, pWWP-luc, has been previously described (19), as has the 3xE2F-luciferase reporter, 3xE2F-Luc, containing three specific E2F DNA-binding sites (50). The cyclin G-luciferase reporter, pGL3-cyclin G-Luc, was obtained from Carol Prives. The β-galactosidase reporter plasmid, pCMX-β-Gal, was used as a control for transfection efficiency (23).

Cells. $INK4a^{-/-}$ MEFs and $ARF^{-/-}$ MEFs have been previously described (37, 58). $RB^{-/-}$ and WT MEFs were prepared from 13.5-day embryos as previously described (32, 76). All MEFs were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% Fetal Clone Serum I (HyClone) and penicillin-streptomycin. MEFs were cotransfected with pEpuro and fivefold excess SV40 TAg-encoding plasmid by the calcium phosphate precipitation method or Fugene 6 (Boehringer Mannheim). After 16 h of exposure to the plasmid DNA, cells were refed with complete medium and grown for 24 h before splitting into medium containing puromycin (2 μg/ml). Before reaching confluence, colonies were pooled and expanded in three 100-mm-diameter plates, at which point they were considered to be established. Cells of no more than three passages after this stage were used for transformation studies and were always passaged at subconfluence.

Western blotting and antibodies. Cells were lysed in high-salt EBC (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 0.5% Nonidet P-40) containing aprotinin (10 μg/ml), leupeptin (10 μg/ml), 0.1 mM phenylmethylsulfonyl fluoride, 4 mM sodium fluoride, and 0.1 mM sodium orthovanadate. Lysates were cleared by centrifugation at 14,000 × *g*, and protein concentration was determined by the Bradford assay (Bio-Rad); 100 μg of each sample was separated in SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked for 1 h with 5% nonfat dry milk and 1% goat serum in TBS-T (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20), before overnight incubation with the primary antibody in TBS-T containing 1% bovine serum albumin at 4°C. The following antibodies were used in this study: anti-TAg polyclonal antibodies (PAb) 101 and 419 (26) (American Type Culture Collection), anti-RB PAb 245 (Pharmingen), anti-p107 C-18 (Santa Cruz Biotechnology), anti-p130 C-20 (Santa Cruz), anti-p53 FL-393 (Santa Cruz), anti-$p15^{INK4b}$ Ab-3 (Neomarkers), anti-$p16^{INK4a}$ M-156 (Santa Cruz), and anti-$p21^{CIP1}$ C-19 (Santa Cruz). The anti-$p19^{ARF}$ rabbit PAb was previously described (37). Detection of proteins was performed with the appropriate horseradish peroxidase-conjugated secondary rabbit or goat antibody (Pierce) at a 1:5,000 dilution in TBS-T containing 2.5% milk and 0.5% goat serum. Immunoblots were developed using enhanced chemiluminescence (Pierce) according to the manufacturer's protocol.

Transformation assays. Soft agar assays were performed in 35-mm-diameter dishes coated with 2 ml of DMEM containing 10% fetal bovine serum (HyClone) and 0.6% agarose (Gibco-BRL). Cells were seeded on top of this layer at a density of 5×10^4 cells per plate in DMEM containing 10% fetal bovine serum and 0.3% agarose. Soft agar colony formation was evaluated 6 weeks after the initial plating. To determine growth to high density, cells were seeded at low density (5×10^4 per 60-mm-diameter dish) and fed every 3 days with DMEM containing 10% fetal bovine serum. Triplicate plates of cells were counted every 2 or 3 days.

Promoter reporter assays. WT and $ARF^{-/-}$ cell lines were plated at a density of 10^5 cells per 35-mm-diameter dish. Cells were transfected the following day with Fugene 6 and 0.5 μg of the appropriate luciferase reporter construct and pCMX-β-Gal (23). At 48 h after transfection, cells were washed twice in phosphate-buffered saline and assayed for β-galactosidase and luciferase activities as previously described (31).

RESULTS

Immortalization of MEFs by SV40 TAg. It has been reported that TAg-mediated transformation of WT MEFs requires the inactivation of the pRB family by the LXCXE motif and J domain and inactivation of p53 function through the p53-binding domain (10, 76, 81). It is possible that these transforming domains of TAg have other functions or affect additional cellular targets. To determine whether these TAg domains contributed to transformation in cells that had undergone inactivation of the pRB and p53 pathways, we used selected knockout mouse strains. $INK4a^{-/-}$ MEFs contain a deletion of the second and third exons of the *INK4a* locus (58). This prevents the expression of both $p16^{INK4a}$ and $p19^{ARF}$, effectively disrupting both the p53 and the pRB pathways. $ARF^{-/-}$ MEFs selectively delete the first exon of the $p19^{ARF}$ protein and retain an intact $p16^{INK4a}$ (37). $RB^{-/-}$ MEFs lose pRB but express the pRB-related proteins p107 and p130 (76).

To determine which domains of TAg are required for transformation of WT, $INK4a^{-/-}$, $ARF^{-/-}$, and $RB^{-/-}$ MEFs, we established cell lines that express WT TAg or various mutants of TAg as depicted in Fig. 1. An intact HPD motif (residues 42

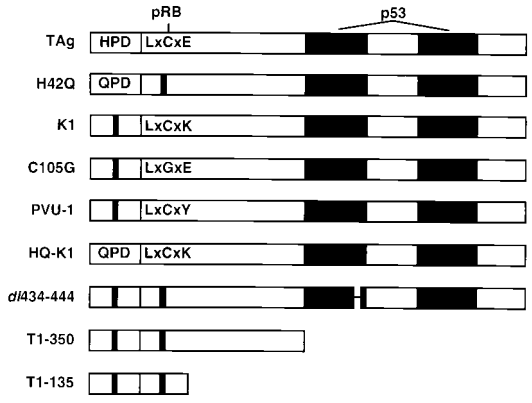

FIG. 1. SV40 TAg constructs. The J domain is contained within the first 82 residues and includes the conserved HPD (residues 42 to 44). The point substitution mutant H42Q inactivates the J domain function of TAg (6, 68). The LXCXE motif is present in residues 103 to 107. The K1, C105G, and PVU-1 mutations disrupt binding to all pRB family members (76). The HQ-K1 mutant contains inactivating mutations in the J domain and LXCXE motif. The *dl*434-444 mutant disrupts the ability of TAg to bind to p53 (40). T1-350 contains the first 350 amino acids of TAg. T1-135 contains the first 131 amino acids of TAg plus 4 additional residues (79). All TAg constructs were expressed as cDNAs in a pSG5 plasmid under an SV40 promoter.

to 44) is essential for J domain function of TAg (38, 65, 68). The H-to-Q point substitution (H42Q) in the HPD motif has been shown to inactivate several J domain functions, including SV40 TAg-mediated *ori*-dependent DNA replication (6). The LXCXE motif of TAg, contained within residues 103 to 107, binds to and inactivates pRB family members (13, 16, 20, 76). The K1 (E107K), C105G, and PVU-1 substitution mutations disrupt the ability of TAg to bind to pRB, p107, and p130. The bipartite p53-binding domain of TAg is located between residues 350 and 550 (40, 51). A small in-frame deletion of residues 434 to 444 completely disrupts the ability of TAg to bind p53 (40). The truncation mutants T1-350 and T1-135 lack the entire p53-binding region but retain an intact J domain and LXCXE motif. All of the SV40 TAg cDNA constructs expressed similar levels of large TAg and did not express small t antigen (68, 76; data not shown).

TAg expression plasmids were cotransfected with a plasmid expressing a puromycin resistance gene (pEpuro) into WT, $ARF^{-/-}$, $INK4a^{-/-}$, and $RB^{-/-}$ MEFs. Early-passage MEFs were transfected to minimize the possibility that the cells had acquired spontaneous mutations. Pools of transfectants were selected in puromycin and tested for TAg expression. Expression of WT TAg or TAg mutants was quite high in all pools of cells and varied less than twofold (data not shown). WT and $RB^{-/-}$ MEFs usually senesce within 10 to 20 passages, and any puromycin-resistant clone was likely to have undergone immortalization (76). In WT MEFs, we observed that only cells that expressed TAg constructs containing an intact p53-binding domain became immortalized, as had been previously reported (40). An intact J domain or LXCXE motif was not required for immortalization. WT MEFs transfected with pEpuro only failed to produce any immortal lines. Similar results were observed with $RB^{-/-}$ MEFs, although a few small colonies expressing either the *dl*434-444 or T1-135 construct were observed (40, 71). Since the $RB^{-/-}$ MEFs expressing the *dl*434-444 construct grew more slowly than cells transfected with other TAg constructs, they were not used in transformation studies. The T1-135 $RB^{-/-}$ MEFs grew slightly faster and were tested in the soft agar assay.

Both $ARF^{-/-}$ and $INK4a^{-/-}$ cells immortalized readily, as determined by the generation of stable pools of puromycin-resistant cells transfected by the puromycin resistance plasmid alone. This observation is consistent with prior reports that $ARF^{-/-}$ and $INK4a^{-/-}$ MEFs were either already immortalized or underwent immortalization very readily (37, 58). Furthermore, the p53-binding domain of TAg was not required for the immortalization of $INK4a^{-/-}$ or $ARF^{-/-}$ MEFs. We were able to generate pools of cells that stably expressed WT TAg or the J domain, LXCXE motif, or p53-binding domain mutants in the $ARF^{-/-}$ and $INK4a^{-/-}$ MEFs. When these cells were examined by indirect immunofluorescence using a monoclonal antibody for TAg, more than 95% of the cells expressed detectable levels of TAg (data not shown). In contrast, when we attempted to generate $ARF^{-/-}$ or $INK4a^{-/-}$ MEFs expressing TAg with mutations in both the pRB-binding and the p53-binding domains, only about 50% of the cells expressed the TAg mutants (data not shown). We were unable to generate populations of $ARF^{-/-}$ or $INK4a^{-/-}$ MEFs that expressed in every cell the K1-*dl*434-444 double mutation or T1-135 or T1-350 containing a mutation in the LXCXE motif (T1-135K1 or T1-350K1). Similarly, a TAg triple mutant in which the J domain, the LXCXE motif, and the p53-binding domain were mutated (H42Q-K1-*dl*434-444) also did not yield clones that expressed TAg in every cell. Although each of these double- and triple-mutant transfectants formed as many colonies as WT TAg transfectants, we found that less than half of the cells lacked detectable TAg expression. Given the lack of uniform expression, $INK4a^{-/-}$ or $ARF^{-/-}$ MEFs transfected with double- or triple-mutant TAg constructs were not studied further.

Role of the LXCXE motif in anchorage-independent growth. To determine the ability of TAg or mutant derivatives to confer anchorage-independent growth, the MEF cell lines were plated in soft agar. Growth was evaluated visually, and colonies containing eight or more cells were scored as transformants (Fig. 2A). WT TAg expression in WT and $RB^{-/-}$ MEFs was capable of inducing growth in soft agar in approximately 40 to 50% of cells seeded. In WT and $RB^{-/-}$ MEFs, the J domain mutant H42Q transformed almost as well as WT TAg, with nearly the same number and size of colonies, indicating that the J domain was not required for anchorage-independent growth (68). In contrast, TAgs containing mutations in the LXCXE motif were unable to transform WT or $RB^{-/-}$ MEFs (68, 76). Thus, an intact LXCXE motif but not the J domain was necessary for the transformation of WT and $RB^{-/-}$ MEFs. Similar results were observed in $p107^{-/-}$, $p130^{-/-}$, and $p107/p130^{-/-}$ MEFs (Zalvide and DeCaprio, unpublished). In each of these genetic backgrounds, the LXCXE mutants failed to induce growth in soft agar, while both WT TAg and the J domain mutant of TAg induced soft agar growth in more than 40% of the cells seeded (data not shown). The truncated T1-135, lacking the p53-binding domain, was able to induce soft agar growth of $RB^{-/-}$ MEFs only slightly more efficiently than the full-length LXCXE mutants, suggesting that the p53-binding domain of TAg may contribute to anchorage-independent growth of $RB^{-/-}$ MEFs.

Since $INK4a^{-/-}$ MEFs disrupt the expression of both $p16^{INK4a}$ and $p19^{ARF}$, these cells are expected to have at least partially inactivated the pRB and p53 tumor suppressor pathways. We therefore reasoned that TAg might not require the LXCXE motif or the p53-binding region to transform $INK4a^{-/-}$ MEFs. As seen in Fig. 2A, WT TAg or mutants containing point substitutions in the LXCXE motif (K1, C105G, or PVU-1), a small in-frame deletion in the p53-binding domain (*dl*434-444), or a deletion of the entire p53-binding domain (T1-135) were able to induce soft agar colony growth

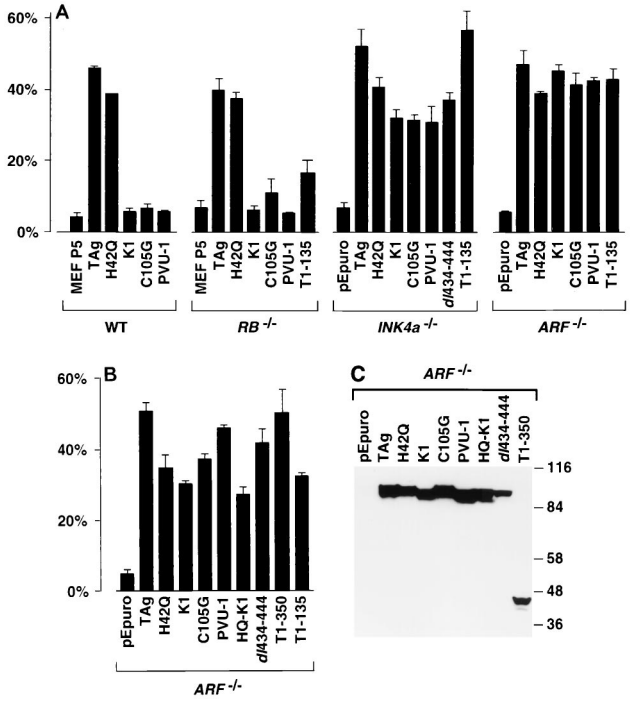

FIG. 2. Ability of TAg to form soft agar colonies in WT, $RB^{-/-}$, $INK4a^{-/-}$, and $ARF^{-/-}$ MEFs. (A) TAg-immortalized MEFs from the indicated cultures were plated in soft agar and allowed to grow for 6 weeks. Colonies containing eight or more cells were scored as positive and indicated as percentage of cells seeded. Nonimmortalized WT and $RB^{-/-}$ MEFs from the fifth passage (P5) were used as controls. Error bars indicate standard deviation. (B) Soft agar colony formation in an independent transformation of $ARF^{-/-}$ MEFs. (C) Western blot of TAg expression in $ARF^{-/-}$ MEFs. Equivalent amount of lysates prepared from each of the indicated MEFs were separated in an SDS–7.5% polyacrylamide gel and blotted with an anti-TAg monoclonal antibody. Sizes are indicated in kilodaltons.

in $INK4a^{-/-}$ MEFs. Notably, some function of TAg was required for transformation of $INK4a^{-/-}$, as the pEpuro-only transfectants were unable to grow in soft agar (Fig. 2A). Thus, expression of TAg containing an intact LXCXE motif or an intact p53-binding region conferred anchorage-independent growth in the $INK4a^{-/-}$ cells.

Surprisingly, $ARF^{-/-}$ MEFs that express $p16^{INK4a}$ but not $p19^{ARF}$ were transformed by the same TAg constructs that were able to transform $INK4a^{-/-}$ MEFs. TAg constructs containing mutations in the J domain (H42Q), the LXCXE motif (K1, C105G, or PVU-1) or the p53-binding domain (T1-135) were able to induce $ARF^{-/-}$ MEFs to grow in soft agar with nearly the same efficiency as WT TAg (Fig. 2A). Since $p19^{ARF}$ was suspected to affect MDM2 and p53 function but not pRB function, the ability of the LXCXE mutants to form soft agar colonies in $ARF^{-/-}$ MEFs was unexpected. However, three different mutant constructs of the LXCXE motif, each unable to bind pRB family members, were able to induce soft agar colony growth as efficiently as WT TAg. Notably, the truncation mutant T1-135, unable to bind to p53, also induced colony formation in soft agar.

To confirm the ability of LXCXE TAg mutants to induce soft agar growth in $ARF^{-/-}$ MEFs, we established a second series of cells with additional TAg constructs. The level of TAg expression was similar in each pool of $ARF^{-/-}$ MEFs, as confirmed by Western blotting (Fig. 2C). Again, the three different LXCXE mutant constructs were each capable of conferring the ability of $ARF^{-/-}$ MEFs to grow in soft agar relative to the pEpuro-only pools of transfectants (Fig. 2B). Furthermore, a J domain-LXCXE double mutant, HQ-K1, was also capable of growth in soft agar. This double mutant, though expected to have completely disrupted any ability to perturb the pRB family of tumor suppressors, was nearly as effective as the K1 and H42Q single mutants in inducing soft agar growth. In addition, three different p53-binding-defective mutants of TAg, *dl*434-444, T1-350, and T1-135, were each able to transform $ARF^{-/-}$ MEFs. Thus, in both $INK4a^{-/-}$ and $ARF^{-/-}$ cells, mutants expressing either an intact p53-binding domain or LXCXE motif were able to induce transformation, as measured by the ability to induce anchorage-independent growth.

Growth to high density requires the J domain and the LXCXE motif. The TAg-transformed phenotype is also reflected in the ability of adherent cells to grow to high density on plastic surfaces. TAg-transformed cells can grow to a higher density than nontransformed cells. To overcome a density arrest, cells must overcome signals for growth arrest due to contact inhibition as well as growth factor depletion. TAg induces high-density growth in cultured cells (76). To determine the domains of TAg that participate in inducing high-density growth, the growth rate of TAg-immortalized MEF cell lines was determined. Cells were plated on a 10-cm-diameter plate at a low density and refed with medium containing 10% serum every 3 days. At various intervals, three replica plates of each transformant were harvested and counted. This growth experiment was repeated at least twice, and the results of a typical experiment are shown in Fig. 3.

WT TAg was able to induce growth to high density in WT MEFs, resulting in approximately a 100-fold increase in cell number after 12 days (Fig. 3A). In contrast, the J domain mutant of TAg (H42Q) grew to approximately half the density as wild-type TAg before undergoing a density arrest. This result suggests that although the J domain had only a very minor influence on the ability of MEFs to grow in soft agar, an intact J domain enhanced the ability of TAg to induce growth to high density. The LXCXE mutants, K1, C105G, and PVU-1, also underwent a density arrest, resulting in three- to fourfold fewer cells relative to WT TAg. The observed contribution of the J domain and LXCXE motif to TAg's ability to overcome density arrest in WT and $RB^{-/-}$ MEFs is entirely consistent with previous reports (68).

WT TAg induced growth to very high density in $INK4a^{-/-}$ MEFs, nearly twice as high as observed in WT MEFs (Fig. 3B). In contrast, the J domain (H42Q) and LXCXE (K1, C105G, and PVU-1) transformants grew to a density half of that observed for MEFs expressing WT TAg but nearly twice that of the pEpuro transformants. The p53-binding-defective mutant *dl*434-444 grew to a slightly lower density than the J domain and LXCXE mutants but still resulted in significantly more cells than the pEpuro-only MEFs. In contrast to the results observed in the anchorage-independent growth assay, loss of the J domain, the LXCXE motif, or the p53-binding domain impaired the ability of $INK4a^{-/-}$ MEFs to grow to high density.

WT TAg induced $ARF^{-/-}$ MEFs to grow to approximately twice the densities of MEFs transfected with the J domain mutant, H42Q, and the LXCXE mutants, C105G and PVU-1 (Fig. 3C). The LXCXE mutant K1 and the J domain-LXCXE double mutant HQ-K1 grew to a slightly lesser extent than the other LXCXE mutants but still grew to a higher density than the pEpuro-only transfectants. Notably, the TAg p53-binding domain mutants *dl*434-444 and T1-350 grew as well as WT TAg, suggesting that the p53-binding domain is not necessary for growth to high density in $ARF^{-/-}$ MEFs. Furthermore, $ARF^{-/-}$ MEFs that expressed T1-135 did not grow to as high

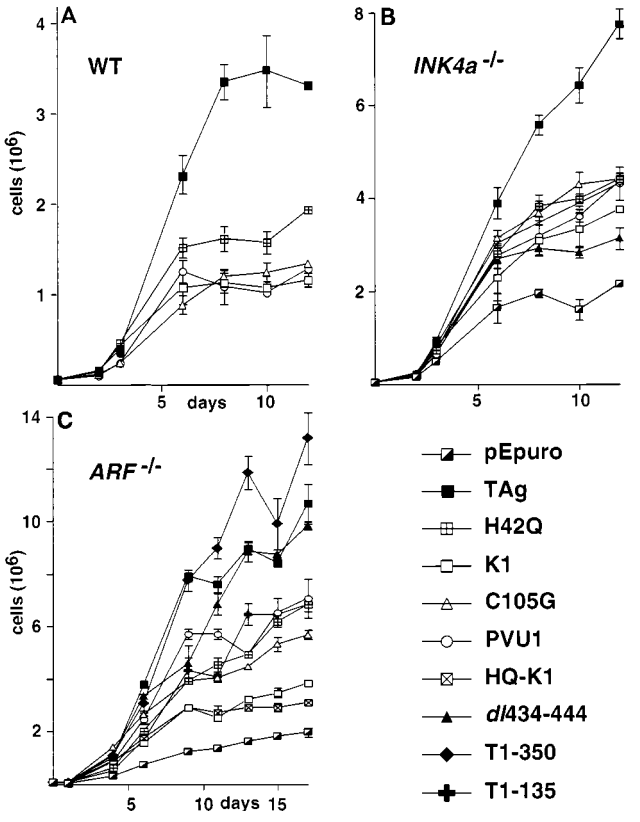

FIG. 3. Ability of TAg-transformed MEFs to grow to high density. Fifty thousand WT, *INK4a*$^{-/-}$, or *ARF*$^{-/-}$ MEFs stably expressing the indicated TAg constructs were seeded on replica 100-mm-diameter plates and fed with complete medium containing 10% serum every 2 to 3 days. Triplicate plates were harvested at the indicated day after seeding, and cells were counted. Error bars indicate standard deviation.

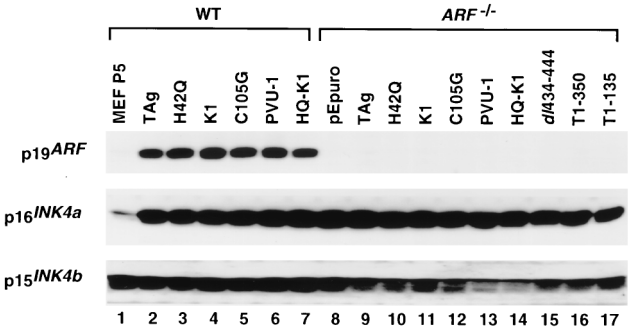

FIG. 4. Western blot for p19^{ARF}, p16^{INK4a}, and p15^{INK4b} in WT and *ARF*$^{-/-}$ MEFs expressing various TAg constructs. Equivalent amounts of lysates prepared from WT and *ARF*$^{-/-}$ MEFs stably expressing the indicated TAg constructs were separated in SDS-polyacrylamide gels and blotted with specific antibodies.

a density as the full-length TAg, T1-350, or *dl*434-444 constructs, suggesting that there may be an additional transforming function within residues 135 to 350 of TAg. Evidence for an additional transforming domain within these residues has previously been proposed (15). The near-wild-type TAg ability of the *dl*434-444 mutant to induce growth in *ARF*$^{-/-}$ MEFs compared to *INK4a*$^{-/-}$ MEFs may be accounted for by the possibility that the *INK4a*$^{-/-}$ MEFs express an N-terminal fragment of the p19^{ARF} protein encoded by the intact exon 1β in the *INK4a* locus (53). The truncated p19^{ARF} may be able to inhibit MDM2 activity and thereby activate p53 (36).

Expression and activity of pRB in *ARF*$^{-/-}$ MEFs. The soft agar experiments shown in Fig. 2A and B suggest that while the LXCXE motif was required for TAg-dependent anchorage-independent growth in WT and *RB*$^{-/-}$ MEFs, it was dispensable in *INK4a*$^{-/-}$ and *ARF*$^{-/-}$ MEFs. This suggests that the loss of p19^{ARF} may have resulted in a partial inactivation of the pRB growth suppression pathway in addition to the p53 pathway. To explore this possibility, we examined the pRB and p53 pathways in WT and *ARF*$^{-/-}$ MEFs.

First, we considered that either the targeted disruption of the 1β exon in the *ARF*$^{-/-}$ MEFs or the stable expression of TAg may have affected expression of the nearby p15^{INK4b} or p16^{INK4a} genes. Loss of either p15^{INK4b} or p16^{INK4a} could lead to an increase in cyclin D-cdk4 activity, resulting in the hyperphosphorylation and inactivation of the pRB family members. However, as shown in Fig. 4, p16^{INK4a} was expressed at relatively similar levels in WT and *ARF*$^{-/-}$ MEFs established by various TAg constructs, and only a slight variation of p15^{INK4b} expression was noted in the *ARF*$^{-/-}$ MEFs. Thus, it is unlikely that the loss of p16^{INK4a} or p15^{INK4b} expression was responsible for the ability of the LXCXE mutant in *ARF*$^{-/-}$ MEFs to overcome the pRB growth suppression pathway in the soft agar assay. Notably, the levels of p16^{INK4a} and p19^{ARF} were low in the early-passage primary WT MEFs and became significantly increased upon TAg-induced immortalization. This increase in expression of p19^{ARF} has been reported during serial passage of WT MEFs (37).

Alternatively, it was possible that loss of p19^{ARF} in *ARF*$^{-/-}$ MEFs affected expression of pRB family members. This was a distinct possibility given the observation that p19^{ARF} affects MDM2 function and MDM2 has been reported to bind to pRB (73). However, as shown in Fig. 5A, there was no significant difference between TAg-expressing WT and *ARF*$^{-/-}$ MEFs in the steady-state levels or phosphorylation state of pRB, p107, and p130. As noted in the introduction, the LXCXE motif and J domain of TAg cooperate to alter the phosphorylation state of p130 and p107 (68, 69). As can be seen in Fig. 5A, the hyperphosphorylated forms of p130 and p107 were not present in the WT or *ARF*$^{-/-}$ MEFs expressing WT TAg (lanes 2 and 9), *dl*434-444 (lane 15), T1-350 (lane 16), and T1-135 (lane 17). In contrast, the hyperphosphorylated forms of p107 and p130 were present in WT and *ARF*$^{-/-}$ MEFs established by the J domain mutant H42Q (lanes 3 and 10), the LXCXE mutant constructs K1 (lanes 4 and 11), C105G (lanes 5 and 12), and PVU-1 (lanes 6 and 13), and the double mutant HQ-K1 (lanes 7 and 14). Thus, the pRB family of proteins were expressed and normally phosphorylated in the presence of LXCXE and J domain mutants of TAg in a manner indistinguishable between WT and *ARF*$^{-/-}$ MEFs.

The E2F transcription factors are among the best-characterized targets of the pRB family. pRB, p107, and p130 bind to and repress the transcriptional activity of certain members of the E2F family (7, 9, 24, 30, 33, 62, 72). To test whether the pRB family retained the ability to repress E2F activity in *ARF*$^{-/-}$ MEFs that expressed TAg or an LXCXE mutant, we examined the activity of an E2F promoter reporter containing three consensus E2F sites (43). As shown in Fig. 6A, WT TAg or the J domain mutant H42Q could transactivate the 3xE2F-Luc reporter relative to non-TAg-expressing WT or *ARF*$^{-/-}$ MEFs. Similarly, the p53-binding mutant T1-135 could also transactivate the 3xE2F-Luc reporter in *ARF*$^{-/-}$ MEFs. In contrast, the activity of the E2F promoter reporter was signif-

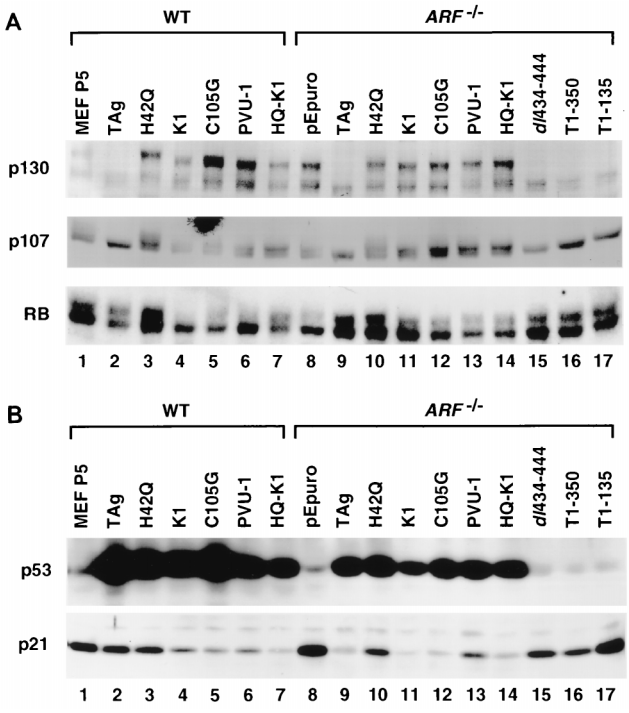

FIG. 5. Expression of pRB family members, p53, and p21 in WT and $ARF^{-/-}$ MEFs. (A) Western blot for pRB, p107, and p130 in WT and $ARF^{-/-}$ MEFs expressing various TAg constructs. (B) Western blot for p53 and p21 in WT and $ARF^{-/-}$ MEFs expressing various TAg constructs.

icantly lower in MEFs established by LXCXE mutants of TAg. The LXCXE mutant K1 was unable to transactivate the 3xE2F-Luc reporter relative to controls in either WT or $ARF^{-/-}$ MEFs. A promoter reporter containing three mutated E2F sites was not affected by expression of TAg or any of the mutated TAg constructs (data not shown). We conclude that the ability of the pRB family to decrease transcription through the E2F site was not abrogated in the LXCXE mutant-expressing $ARF^{-/-}$ MEFs.

Expression and activity of p53 in $ARF^{-/-}$ MEFs. SV40 TAg has a well-described ability to bind to p53 and to increase its level of expression at least in part through decreasing the turnover rate (70). As shown in Fig. 5B (top panel), p53 expression was extremely low in the pEpuro $ARF^{-/-}$ MEFs (lane 8) as well as in early-passage cultures of WT MEFs (lane 1). In contrast, WT and $ARF^{-/-}$ MEFs expressing WT TAg (lanes 2 and 9), LXCXE mutants (lanes 4 to 6 and 11 to 13), or J domain mutants (lanes 3, 7, 10, and 14), which are capable of binding to p53, had significantly increased levels of p53. This observation suggests that TAg could stabilize p53 both in WT cells and in cells that have lost $p19^{ARF}$. In contrast, the expression of p53-binding mutants *dl*434-444 (lane 15), T1-350 (lane 16), and T1-135 (lane 17) did not result in increased steady-state levels of p53.

To determine whether p53 retained the ability to transactivate certain promoters in $ARF^{-/-}$ MEFs, we measured the activity of two p53-responsive promoter reporters, cyclin G and p21, in WT and $ARF^{-/-}$ MEFs. As shown in Fig. 6B, WT TAg and C105G, an LXCXE mutant, strongly repressed the transcription of the cyclin G promoter relative to the activity observed in the pEpuro-transfected $ARF^{-/-}$ MEFs. In contrast, p53-binding mutants of TAg, *dl*434-444 and T1-350, failed to repress the activity of the cyclin G promoter. Similar results were observed with the $p21^{CIP}$ promoter. WT TAg, the J domain mutant H42Q, and two different LXCXE mutants repressed the activity of the $p21^{CIP}$ promoter, while the p53-binding mutant *dl*434-444 was unable to repress this promoter. The levels of $p21^{CIP}$ protein seen in TAg-expressing WT and $ARF^{-/-}$ MEFs reflected the changes seen in promoter activity (Fig. 5B, lower panel). $p21^{CIP}$ levels were high in WT MEFs and in the $ARF^{-/-}$ pEpuro-only transfectants (lanes 1 and 8). $p21^{CIP}$ levels were also quite high in the $ARF^{-/-}$ cell lines expressing any of the p53-binding mutants of TAg, *dl*434-444

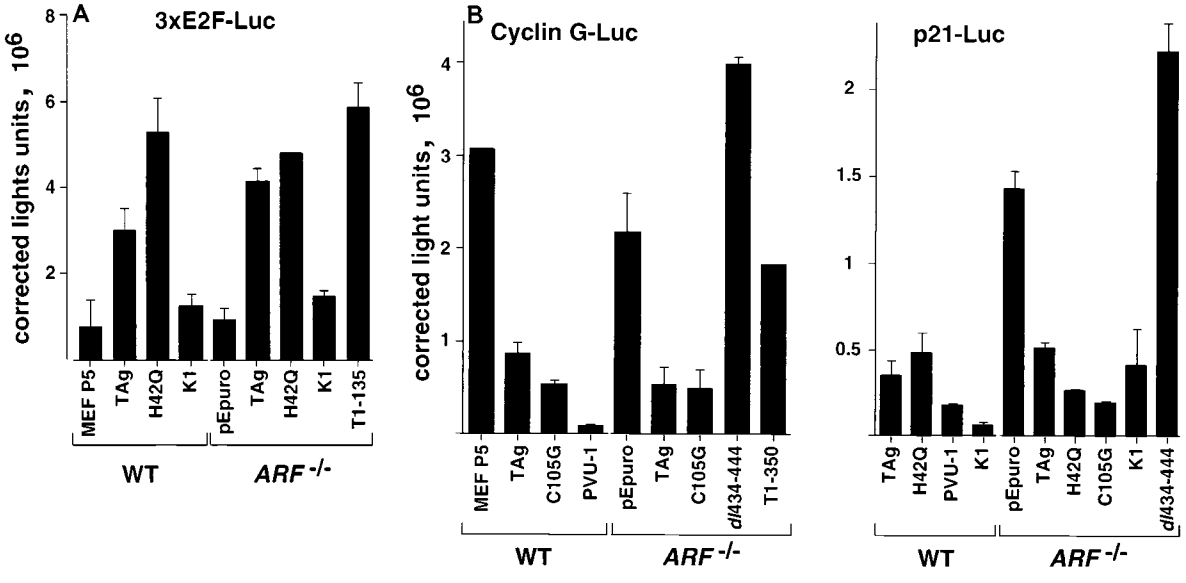

FIG. 6. E2F and p53 promoter reporter activity in TAg-transformed WT and $ARF^{-/-}$ MEFs. The reporter constructs (A) 3xE2F-Luc and pCMV-β-Gal were cotransfected into the indicated stable TAg-transformed MEFs. Luciferase activity was normalized to β-galactosidase activity and expressed as relative luminometer units. The experiment was performed in triplicate; error bars indicate standard deviation. (B) Cyclin G promoter-luciferase reporter (left) and p21 promoter-luciferase promoter (right) activities were determined as described above.

(lane 15), T1-135 (lane 16), or T1-350 (lane 17). These results suggest that in $ARF^{-/-}$ MEFs stably expressing p53-binding mutants of TAg, p53 retained the ability to act as a DNA-specific transcription factor.

DISCUSSION

In this report, the contribution of the J domain, LXCXE motif, and p53-binding domain of TAg to transformation of WT, $RB^{-/-}$, $INK4a^{-/-}$, and $ARF^{-/-}$ MEFs was examined. The goal was to determine whether the loss or functional inactivation of certain tumor suppressor genes would reduce the requirement for these domains in TAg transformation. WT SV40 TAg was able to induce growth of MEFs derived from each of these genetic backgrounds to high density and in an anchorage-independent manner. In WT and $RB^{-/-}$ MEFs, TAg's p53-binding domain was required for efficient immortalization, the LXCXE motif was needed for anchorage-independent and high-density growth, and the J domain was required for high-density growth. In contrast, TAg had different requirements for transformation of MEFs that did not express $p16^{INK4a}$ or $p19^{ARF}$. The p53-binding domain of TAg was not required for immortalization, high-density growth, or soft agar growth of $INK4a^{-/-}$ or $ARF^{-/-}$ MEFs. These results were not unexpected given that the loss of $p19^{ARF}$ expression has been shown to perturb the p53 growth suppression pathway, thereby reducing the requirement for p53 inactivation by TAg binding. The LXCXE motif and J domain were required for high-density growth of $INK4a^{-/-}$ MEFs, suggesting that the pRB family was functionally intact despite the loss of $p16^{INK4a}$ expression. The most unexpected observation was that the LXCXE motif was not required for anchorage-independent growth of either $INK4a^{-/-}$ or $ARF^{-/-}$ MEFs. This latter result would seem to suggest that the growth suppression functions of the pRB family were disrupted in $ARF^{-/-}$ and $INK4a^{-/-}$ MEFs during anchorage-independent growth. Notably, the $ARF^{-/-}$ MEFs which expressed $p16^{INK4a}$ but not $p19^{ARF}$ behaved similarly as $INK4a^{-/-}$ MEFs in response to TAg. Both cell types were induced to grow in soft agar when expressing either p53-binding domain or LXCXE motif mutants of TAg. Since $p19^{ARF}$ was reported to regulate the p53 pathway but not the pRB pathway, the LXCXE mutants were not expected to induce anchorage-independent growth in $ARF^{-/-}$ MEFs that expressed $p16^{INK4a}$.

It is unlikely that $INK4a^{-/-}$ and $ARF^{-/-}$ MEFs expressing the LXCXE mutant TAg constructs have acquired additional specific genetic modifications that enabled them to grow in soft agar. All assays were performed with pools of transfected cells to reduce the possibility of clonal variation. Furthermore, similar transformation results were obtained with several independently performed transfections. $INK4a^{-/-}$ or $ARF^{-/-}$ MEFs could be readily immortalized in the absence of TAg, as evidenced by the efficient ability to select for colonies in the presence of the puromycin resistance gene alone. However, $INK4a^{-/-}$ or $ARF^{-/-}$ MEFs expressing the puromycin resistance gene alone were not transformed, as determined by their inability to grow in soft agar or to high density. Expression of TAg conferred an additional transforming activity that permitted growth under these more stringent conditions. These observations are consistent with the demonstration that expression of activated H-*ras* induced transformation of $ARF^{-/-}$ and $INK4a^{-/-}$ MEFs (37, 58).

Growth in soft agar measures the ability of fibroblasts to acquire anchorage-independent growth. Anchorage-independent growth has been considered a stringent in vitro method for predicting the tumor formation potential of TAg-transformed fibroblasts (55). However, it has been reported that the ability of TAg-transformed cells to form tumors in nude mice does not require an intact LXCXE motif (71). To date, there has been an absolute requirement for an intact LXCXE motif in anchorage-independent growth of TAg-transformed WT MEFs in soft agar (10, 11, 71, 76). Here, we observed that the LXCXE motif was not required for soft agar growth of $INK4a^{-/-}$ and $ARF^{-/-}$ MEFs.

Notably, expression of HQ-K1, a TAg containing inactivating mutations in both the LXCXE motif and J domain, induced $INK4a^{-/-}$ or $ARF^{-/-}$ MEFs to grow in soft agar. Despite loss of the J domain and LXCXE motif, HQ-K1 could bind to and stabilize p53. Transformation by this and other LXCXE mutants would seem to indicate that binding to p53 conferred a growth advantage and that p53 function was not completely eliminated by the loss of $p19^{ARF}$. Consistent with this possibility, p53 appeared to be functional in the $ARF^{-/-}$ MEFs that express TAg with mutations in the p53-binding domain, as demonstrated by the increased activity of the $p21^{CIP}$ and cyclin G promoters (Fig. 6B) as well as by the increased expression of $p21^{CIP}$ (Fig. 5B). This potential for activation of p53 in $ARF^{-/-}$ MEFs did not seem to pose a barrier to immortalization or to the ability of the p53-binding mutant TAgs to induce growth in soft agar or to high density.

The ability of adherent cells to grow to high density may reflect the cell's response to limiting amounts of growth factors and nutrients as the cell number increases. An intact J domain and LXCXE motif were both required for TAg to induce growth to high density in WT, $RB^{-/-}$, $ARF^{-/-}$, and $INK4a^{-/-}$ MEFs (Fig. 3 and reference 68). The contribution of the J domain to high-density growth of these MEFs is consistent with previous work that suggests that the J domain cooperates with the LXCXE motif to inactivate pRB, p107, and p130 (27, 60, 68). Further evidence for the J domain activity includes the reduction in phosphorylation of p107 and p130 in WT and $ARF^{-/-}$ MEFs expressing TAg (Fig. 5B). We have previously reported that J domain mutant constructs were as effective as WT TAg in promoting the high-density growth of $p130^{-/-}$ $p107^{-/-}$ double-knockout MEFs, suggesting that the J domain specifically contributed to the functional inactivation of p107 and p130 (68). The observation that the J domain and the LXCXE motif were required for overcoming density arrest of $INK4a^{-/-}$ and $ARF^{-/-}$ MEFs suggests that the pRB family including p107 and p130 was functionally intact in adherent $INK4a^{-/-}$ and $ARF^{-/-}$ MEFs. Therefore, loss of $p16^{INK4a}$ or $p19^{ARF}$ expression did not perturb the pRB growth suppression activity in the density arrest assay. It will be interesting to determine whether TAgs containing J domain or LXCXE motif mutants will have the ability to induce high-density or soft agar growth in MEFs homozygously deficient in *Rb*, *p107*, and *p130*.

The J domain cooperates with the LXCXE motif to inactivate pRB function in a variety of assays including dissociation of pRB-E2F complexes and overriding pRB repression of E2F-dependent promoters (68, 77). Recent reports suggest that the LXCXE motif of SV40 and polyomavirus large T antigens may also inhibit certain pRB functions independently of the J domain. For example, WT TAg and a J domain mutant (H42Q) but not an LXCXE mutant (K1) could override a p53-induced growth arrest (25). The LXCXE motif but not the J domain was required for polyomavirus large T antigen-induced apoptosis of C2C12 myoblasts upon serum withdrawal (61). In this report, we observed that an intact LXCXE motif but not the J domain was required for activation of the 3xE2F promoter reporter in WT and $ARF^{-/-}$ MEFs (Fig. 6A). In addition, an intact J domain was not required for inducing soft agar growth

of MEFs derived from any of the genetic backgrounds studied. These distinctions may reflect the ability of TAg to perturb different growth-suppressing functions of the pRB family. Alternatively, the LXCXE motif may target other cellular proteins that do not require the contribution of the J domain.

As mentioned earlier, the most unexpected observation was that the LXCXE mutants of TAg could induce anchorage-independent growth of $ARF^{-/-}$ MEFs. There are several models to explain how the LXCXE mutant TAgs were able to confer anchorage-independent growth of the $ARF^{-/-}$ and $INK4a^{-/-}$ MEFs. One possibility is that $p19^{ARF}$ is involved in the regulation of both p53 and pRB and that loss of $p19^{ARF}$ led to at least partial deregulation of both pathways. If this were true, it could explain why $ARF^{-/-}$ mice display the same tumor types as $INK4a^{-/-}$ mice. Several reports suggest that $p19^{ARF}$ may regulate pRB function. For example, the expression of an antisense construct to $p19^{ARF}$ could overcome a growth arrest induced by $p16^{INK4a}$ whereas the expression of an antisense construct to $p16^{INK4a}$ was unable to overcome a growth arrest induced by $p19^{ARF}$, suggesting that $p19^{ARF}$ has functions that overlap with those of $p16^{INK4a}$ (8). In addition, overexpression of $p19^{ARF}$ was able to induce a growth arrest in $p53^{-/-}$ cells but not in $p53^{-/-}$ cells that overexpressed E2F-1, again suggesting that $p19^{ARF}$ may be able to suppress growth through regulation of pRB as well as p53 (8). In addition, MDM2 may be directly involved in the regulation of pRB by binding to the C-terminal region of pRB (73). Alternatively, MDM2 may promote the rapid degradation of E2F-1 in cells that lack $p19^{ARF}$ (4). Under such conditions, it is likely that pRB would be at least partially unable to act as a tumor suppressor gene, since pRB binding to E2F-1 is required for transcriptional repression of E2F-dependent promoters and tumor suppression (22, 75). However, our data do not support such a scenario since we were unable to detect any differences in E2F activity between WT and $ARF^{-/-}$ MEFs transformed by LXCXE mutants of TAg. Furthermore, the steady-state levels of E2F-1 appeared similar between the WT and $ARF^{-/-}$ cells as determined by Western blotting (data not shown). We have not extensively tested the ability of the pRB family to bind and inactivate other members of the E2F family in the TAg-transformed MEFs. It is possible that the activities of one or more members of the E2F family are diminished in $ARF^{-/-}$ MEFs. Alternatively, it is possible that the LXCXE region of TAg targets an additional growth or tumor-suppressing protein whose activity may be lacking or diminished in $ARF^{-/-}$ MEFs.

The ability of the LXCXE mutant constructs to induce anchorage-independent growth in $ARF^{-/-}$ MEFs may also reflect a novel transforming function of TAg. This additional activity could overcome the growth inhibition induced by the pRB family. p300 is an especially interesting candidate for this additional activity. p300 was originally cloned as an adenovirus E1A-associated protein (18), and both p300 and the highly related CREB-binding protein (CBP) can be functionally inactivated by E1A (1, 49). E1A binds directly to p300 and CBP through a domain distinct from its pRB family-binding motif. E1A can stimulate entry into the cell cycle from quiescence using either the p300/CBP-binding region or the pRB-binding LXCXE motif (66, 78). Thus, E1A binding to p300/CBP can circumvent at least some of the growth-inhibitory functions of pRB. The mechanism of the E1A/p300 growth-promoting activity, however, is not completely understood. The effect of p300 binding to TAg also remains unknown; however, it is possible that this permits TAg to overcome the growth-suppressing function of the pRB family in $ARF^{-/-}$ MEFs (2, 45). The p53-binding domain of TAg can also bind to MDM2 (29). Thus, TAg could perturb the function of several growth-regulatory factors, including p53, MDM2, p300/CBP, and pRB.

In addition to the three TAg domains described here, it is possible that TAg has additional domains that contribute to transformation. The N terminus of TAg is known to bind to other cellular proteins in addition to the pRB family, most notably a 185-kDa protein that is as yet uncharacterized (42). In addition, there has been some suggestion that the DNA-binding domain of TAg (residues 131 to 280) may contribute to growth promotion and transformation. The DNA-binding region is involved in transcriptional transactivation of many cellular and viral promoters that may promote cellular growth. For example, a point substitution mutation in the DNA-binding domain reduced the ability of TAg to induce DNA synthesis in quiescent cells (15, 34). In our experiments, $ARF^{-/-}$ MEFs expressing T1-135 grew to half the density of those expressing full-length TAg, T1-350, or *dl*434-444 (Fig. 3). This may indicate that the DNA-binding region (residues 131 to 280) or perhaps the zinc finger (residues 302 to 320) can contribute to TAg-mediated transformation, though the nature and extent of this transforming activity are as yet uncharacterized.

In conclusion, these experiments have demonstrated that mutations in either the p53-binding domain or the LXCXE motif did not diminish the ability of TAg to confer anchorage-independent growth of $INK4a^{-/-}$ and $ARF^{-/-}$ MEFs. These results support the notion that the N-terminal and C-terminal regions of TAg have transforming functions beyond the disruption of the pRB and p53 tumor suppressor pathways. Furthermore, these results suggest that $p19^{ARF}$ may have tumor suppression functions in addition to control of the p53 tumor suppressor gene.

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